TITLE:

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AUTHOR(S):

YOSHINAGA, MICHIO

CITATION:

YOSHINAGA, MICHIO. Experimental Studies on the Initiating Factor of Cholesterol Gallstones, Especially on the Influence of Essential Fatty Acids and Pyridoxin on the Bile Constituents. 日本外科宝函 1965, 34(1): 1-18

ISSUE DATE:

1965-01-01

URL:

http://hdl.handle.net/2433/206455

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Experimental Studies on the Initiating Factor of Cholesterol Gallstones, Especially on the Influence of Essential Fatty Acids and Pyridoxin on the Bile Constituents

by

MICHIO YOSHINAGA

From the 2nd Surgical Division, Kyoto University, Medical School
(Director : Prof. CHUJI KIMURA)
Received for Publication Nov. 11, 1964

I INTRODUCTION

Miscellaneous concepts on the initiating factors of gallstone formation originated in an inflammatory theory advocated by NAUNYN (1892) and in a non-inflammatory bile stasis theory by ASCHOFF-BACMEISTER (1909). Thereafter, LICTWITZ (1907) and SCHADE (1910) tried to elucidate more reasonably the initiating factors of gallstone formation with their colloid-chemical theories. Many other investigators declaired the various theories that etiology of gallstones might be attributed to dysfunctions of the endocrine system, dysharmonies of the autonomic nervous system (WESTPHAL 1923, BERG 1923), abnormalities of the constitution (v. BERGMANN JR. 1926) and so on.

It has been said that a series of phenomenons in bile constituents,— decrease of the bile salts and lecithin, appearance of free bile acids, lowering of pH, increase of calcium and etc. —, induced a break-down on bile-equilibrium and then gallstones occurred.

MIYAKE reported that these phenomenons in bile were induced due to bacterial infection, inflammation of the gallbladder, bile stasis or disturbance of liver function. And LARGE declared that minimal damage to the gallbladder wall, cause of which was most likely infection of one form or another, was the initiating mechanism in gallstone formation.

On the other hand, MATUO succeeded in the formation of experimental gallstones in rabbits, which were kept on fat soluble vitamin deficient diet or lanolin diet, treated with the over-administration of vitamin D or the successive administration of sulphurous powder, or extirpation of left adrenal. Therefore, MATUO insisted that gallstones could be formed due not only to local factor but also to disturbance of metabolism, namely to the systemic factor. Also, ANDERSON reported the pure gallstones were formed due rather to disturbances of metabolism or of liver function than to an inflammatory reaction of the gallbladder or of the biliary ducts.

HIKASA et al. verified that a great quantity of essential fatty acids (abridged as EFA) were contained in liver, adrenals and heart. Moreover, HIKASA, FUKUDA and MURAOKA verified that not quantity of cholesterol in adrenals, but quality of fatty aicds combined with cholesterol exerted an influence on adrenocortical capacity ; namely, cholesterol metabolism toward glucocorticoid was concerned directly with quantity and quality of EFA. It has been indicated that bile acids, changes of which was one of the important factors on

gallstone formation, was the other end-metabolite of cholesterol. Therefore, it may be more reasonable to consider that cholesterol metabolism toward bile acid is related to the deficiency or metabolic disturbance of EFA, and thus metabolic disturbances of EFA play a primary role on gallstone formation.

II EXPERIMENTAL ANIMALS AND METHODS

(A) Experimental Animals

Male albino rats of the Wistar strain supplied by the Animal Center of Kyoto University were used. The weanling rats were fed a standard diet (rat chow, produced by ORIENTAL Yeast Ind., Co. Ltd., Japan) until their body weight reached 40 to 50 g and then were divided into 5 groups, each of which consisted of 5 to 6 rats; (1) EFA diet (2) EFA deficient diet (3) Vit. B_6 deficient EFA diet (4) Vit. B_6 deficient EFA with lard diet (5) magnesium deficient EFA diet.

Each of (1)-and (2)-diet group was fed each diet as shown in Table 1, 3 and 4 for 12 weeks. (3)-, (4)- and (5)-diet group were fed EFA diet for 4 weeks and then each

Table 1 Composition of the Diets

	EFA Deficient Diet	EFA Diet	EFA with Lard Diet
Starch	80.0% (w/w)	65.0% (w/w)	65.0% (w/w)
Vitamin-free Casein	16.0	16.0	16.0
Salt Mixture	3.0	3.0	3.0
Vitamin Mixture	0.5	0.5	0.5
Cholin Chloride	0.5	0.5	0.5
Sesame Oil	—	15.0	5.0
Lard	—	—	10.0

(1) In Vit. B_6 deficient diet, Vit. B_6 free vitamin mixture was used.
(2) In magnesium deficient diet, magnesium free salt mixture was used.

Table 2 Composition of Fatty Acids in Sesame Oil and Lard

Fatty Acid	Sesame Oil	Lard
14 : 0	—(%)	2.0*(%)
15 : 0	—	0.3
16 : 0	9.5	28.1
16 : 1	0.7	3.0
17 : 0	—	1.0
18 : 0	5.1	12.5
18 : 1	31.6	41.9
18 : 2	48.9	6.7
18 : 3	0.7	0.3
20 : 0	0.5	1.0
20 : 4	—	—

* : Percentage of total area of gas-liquid chromatographic elution diagram

Table 3 Vitamin Mixture per 1 g

Vit. A	2500 I.U.
Thiamine Nitrate	1.0mg
Riboflavin	1.5 〃
Pyridoxin Hydrochloride	1.0 〃
Cyano-cobalamin	1.0γ
Ascorbic Acid	37.5mg
Calciferol	200 I.U.
dl-α-Tocopherol	1.0mg
Vit. K	0.2 〃
Nicotinic Amid	10.0 〃
Pantothenic Cal.	2.5 〃
Folic Acid	0.5 〃

Table 4 Salt Mixture per 1 kg

NaCl	46.3g
NaH_2PO_4	92.0
K_2HPO_4	253.0
$CaH_4(PO_4)_2H_2O$	143.0
Cal. Lact.	369.0
$MgSO_4$	70.4
KI	26.3

of them was fed respectively each specific diet as shown in Table 1, 3 and 4 for 8 weeks. After the above stated periods elapsed, the specific skin findings were recognized in the deficient groups. These rats were housed in single cages and carefully kept in a room maintained at a constant temperature of 20°C.

The source of EFA used was a purified and peroxide-free sesame oil with a linoleic acid content of 48.9% (Table 2). The lard used contained cholesterol of 0.17%. Magnesium deficient EFA diet contained only 23 ppm of magnesium in comparison with the magnesium content of 426 ppm in the EFA diet.

(B) Experimental Methods

(1) Collecting Method of Hepatic Bile

Under intraabdominal anesthesia of 0.5% nembutal, polyethylene tube was inserted into the proxymal 1/3 of the common bile duct to avoid the mixture of pancretic juice, proceeded to the bifurcation of the hepatic ducts and it was anchrosed by fine silk ligatures there. The rats operated were housed in single cages, and the hepatic bile was collected aseptically for 24 hrs. into each small glass-bottle fixed on their back. The bile collected was used immediately for biochemical analysis of bile acids, cholesterol and fatty acids.

(2) Extraction of Bile Acids

Bile acids were extracted by an adaptation of the procedure reported by MOSBACH et al.. An aliquot of bile (usually 2.0 ml) collected aseptically was added with 5 volumes of absolute ethanol, the solution was heated to boiling on the steam bath, and filtered. The solution was extracted twice with equal volumes of petroleum ether to remove neutral lipids (cholesterol). The alcohol phase was then evaporated on the steam bath under a current air. The residues were dissolved into 5% NaOH and hydrolyzed by autoclaving at 120°C for 3 hrs..

The hydrolysates were acidified with 3 N-HCl and extracted four times with 10 ml of ethyl ether. The ether extracts were washed to remove chlorides and dried with anhydrous Na_2SO_4. The ether extracts were divided into two portions to determine the bile acids.

(3) Determination of Bile Acids by Spectrophotometry

Ether was evaporated on the steam bath and the residues were dissolved into acetone and a suitable aliquot of this solution was pipetted into a tube. Acetone was evaporated on the steam bath under a current air. Five milliliters of 65% sulfuric acid was added to each tube, and the samples were heated at $60 \pm 1^{\circ}C$ (water bath) for 15 min.. After the tube was cooled at room temperature for 15 min., absorption determinations were made at 3200 and 3850 Å with the BECKMAN spectrophotometer. Bile acid concentration was caluculated as described by Gibb.

(4) Determination of Bile Acids by Gas-liquid Chromatography

Bile acids in ether were methylated with freshly prepared diazomethane, and ether was then removed by evaporation under a stream of nitrogen. To the methyl esters of

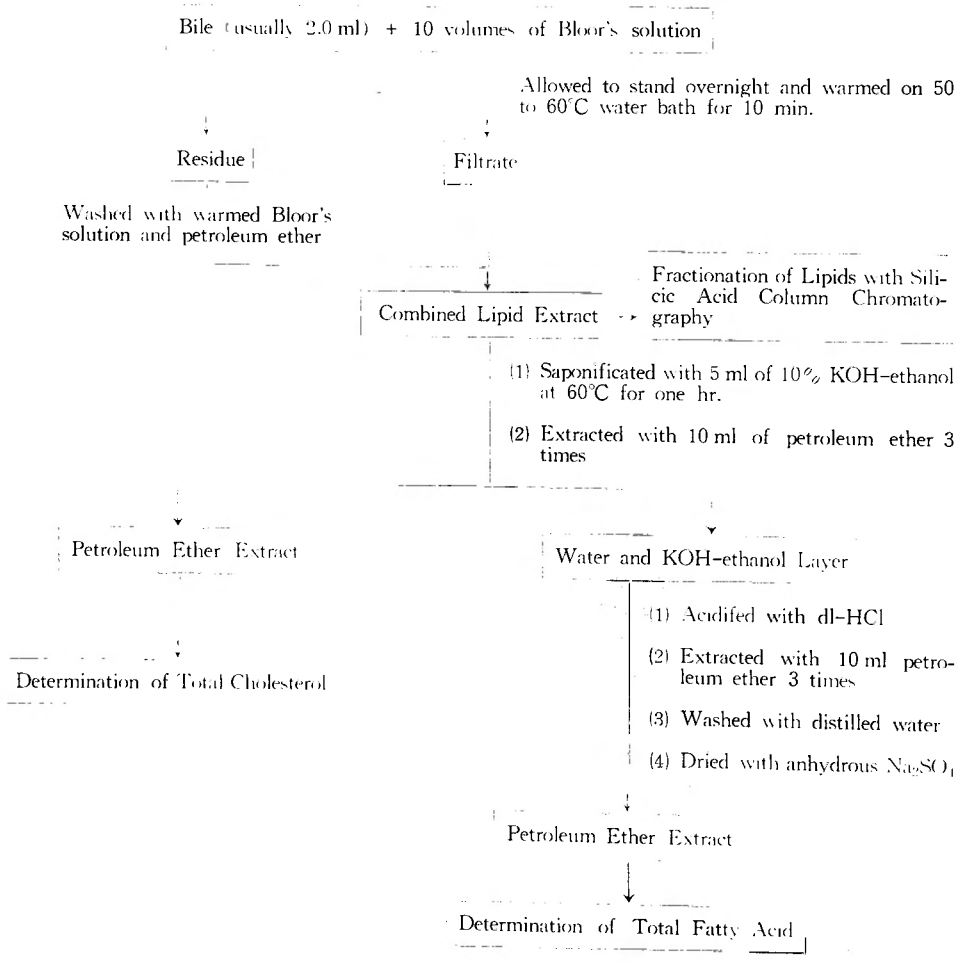
bile acids (ca. 10 mg) were added 1.0 ml of tetrahydrofuran, 0.1 ml of trifluoroacetic anhydride and 0.1 ml of pyridine, and then by heating them at 30°C for 15 min., trifluoroacetates were produced. The reagent was evaporated under a stream of nitrogen and the residues were extracted three times with 5 ml of ethyl ether. The ether extracts were washed with dil-HCl and then water, and dried with anhydrous Na_2SO_4. Ether was evaporated and the residues were dissolved into acetone.

The trifluoroacetates of bile acids in aceton solution were chromatographed in a SHIMADZU Model GC-1B gas-liquid chromatograph equipped with a hydrogen flame ionization detector. A 225 cm × 4 mm i. d. U-shaped column packed with 0.7% nitril-silicon coated on Chromosorb W (60 to 80 mesh) was used. The flash heater was maintained at 230 to 240°C, the column at 230°C, and the detector at 240°C; the nitrogen flow rate was 30 ml/min. × 2, the gas pressure 3 kg.

(5) Extraction of Other Lipids than Bile Acids

Regard to the extraction of other lipids than bile acids, there was no distinct difference among modified BLOOR's method, NODA-HIRAYAMA's method and FOLCH's method, therefore, in this study, modified BLOOR's method was used as shown in Fig. 1.

Fig. 1 Extraction of other Bile Lipids than Bile Acids



(6) Fractionation of Bile Lipids with Silicic Acid Column Chromatography

In fractionating of bile lipids, the modified HIRSH and AHRENS' method was used. Petroleum ether containing 1, 10 and 20% (v/v) of ether, which consisted of 80, 100 and 150 ml respectively, was used to elute successively cholesterol esters, triglycerides and cholesterol. Phospholipids (lecithin) were subsequently eluted with 300 ml of methanol. Cholesterol esters, triglycerides, and phospholipids (lecithin) were saponificated, acidified and extracted with petroleum ether as mentioned earlier. Thereafter, cholesterol and fatty acids were determined as follows.

(7) Determination of Cholesterol

Petroleum ether was evaporated and the residues dissolved into suitable chloroform. According to modified KINGSLEY's method, 5 ml of chloroform solution was pipetted into the tube and mixed with 2 ml of freshly prepared reagent (4 volumes of acetic anhydride : 1 volume of concentrated sulfuric acid). Maximum colored point was read on the BECKMAN spectrophotometer using 620 mμ as the absorption beam.

(8) Determination of Fatty Acids

After adding hydroquinone to avoid oxidation, petroleum ether was evaporated under a stream of nitrogen and fatty acids weighed in the microbalnce after desicating for 12 hrs.. Fatty acids were methylated with freshly prepared 2% H_2SO_4-methanol on 70°C water bath for 90 min.. The methyl esters of fatty acids in petroleum ether were chromatographed in a SHIMADZU Model GC-1B gas-liquid chromatograph equipped with a hydrogen flame ionization detector. A 150 cm × 6 mm i. d. U-shaped column packed with 25% diethylene glycol succinate on Shimalite (60 to 80 mesh) was used. The flash heater was maintained at 280 to 300°C, the column at 210°C, and the detector at 240°C ; the nitrogen flow rate was 30 ml/min. × 2, the gas pressure 3 kg.

Each component of fatty acids separated by gas-liquid chromatography was identified, respectively, with the standard samples obtained from National Institute of Health and Hormel Institute in U. S. A..

III RESULTS

Mean body weights, when polyethylene tube inserted, were 190.6 g in EFA diet group, 144.3 g in EFA deficient diet group, 152.1 g in Vit. B_6 deficient EFA diet group, 208.3 g in Vit. B_6 deficient EFA with lard diet group, and 188.0 g in magnesium deficient EFA diet group. In this study, EFA diet group was taken as control.

(1) Cholesterol and Bile Acids

Cholesterol in bile of rats consisted of free form in more than 98% and only traces of esterified form. Cholesterol showed a tendency to increase, but statistically it was not evident, in other groups than Vit. B_6 deficient EFA with lard diet group, in which was observed almost same value as control group (Table 5).

It was made sure with the aid of gas-liquid chromatography that the major components of the bile acids in bile of rats were cholic and chenodeoxycholic acid as reported previously by many investigators (Fig. 2).

In EFA deficient diet, Vit. B_6 deficient EFA diet, and Vit. B_6 deficient EFA with lard diet groups, total bile acids and ratio of total bile acids to cholesterol (abridged as

Table 5 Cholesterol and Bile Acids in Bile of Rats Fed Various Diets

	Cholesterol (mg/dl)	Total Bile Acids (mg/ml)	Ratio of Dihydroxycholanic Acid to Trihydroxycholanic Acid	Ratio of Total Bile Acids to Cholesterol
(1) EFA Diet	9.9±0.8*	4.15±0.30	0.43±0.10	12.8±7.6
(2) EFA Deficient Diet	12.6±1.6	2.37±0.29	0.75±0.09	20.9±3.6
(3) Vit. B_6 Deficient EFA Diet	12.8±0.6	1.94±0.25	0.93±0.12	15.5±2.3
(4) Vit. B_6 Deficient EFA with Lard Diet	9.3±1.5	1.92±0.09	1.31±0.31	21.6±2.9
(5) Mg Deficient EFA Diet	10.6±1.0	2.61±0.19	0.52±0.07	25.6±3.6

* : Standard error

B/C-ratio) decreased significantly ; ratio of dihydroxycholanic acid to trihydroxycholanic acid (abridged as Di/Tri-ratio) increased moderately. In magnesium deficient EFA diet group, total bile acids and B/C-ratio decreased moderately, and Di/Tri-ratio increased slightly (Table 5).

Saying briefly these results, the same quantitative and qualitative changes of the bile acids as observed in the patients with gallstones occurred in all kinds of diet group. And it is an important fact that these changes were observed not only in EFA deficient diet group, but also in Vit. B_6 deficient EFA diet group and in magnesium deficient EFA diet group. In other words, it will be sure that deficiency and metabolic disturbance of EFA have disturbed the cholesterol degradation toward bile acids.

(2) Fatty Acids

BLOMSTRAND and MARUYAMA reported that fatty acids of bile lecithin represented about 95 to 99 per cents of total fatty acids in human bile. Also in bile of rats, it was assured with the aid of silicic acid column chromatography that more fatty acids than 95 per cents of total fatty acids consisted of lecithin composing fatty acids. And as indicated in Table 6, the detailed composition of total fatty acids showed almost the same percentages as that of lecithin composing fatty acids ; also in human bile, the same result was pointed out by NISHIMURA and MARUYAMA.

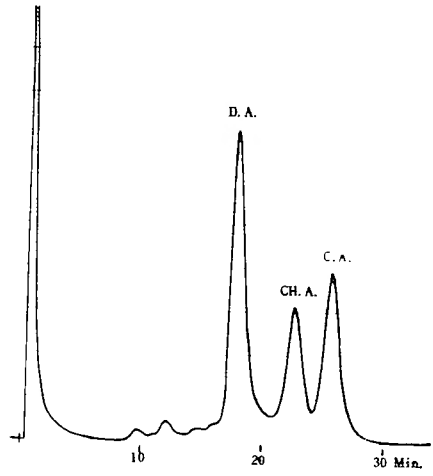
Total fatty acids, namely lecithin, in bile decreased moderately in EFA deficient diet group and in Vit. B_6 deficient EFA with lard diet group, especially in latter diet group. In other diet groups, a tendency of very slight decrease was observed. However, no qualitative difference of total fatty acids, namely of lecithin composing fatty acids, was observed among other four diet groups (including control group) than EFA deficient diet group. In these diet group, main fatty acids were palmitic acid, oleic acid, linoleic acid, and arachidonic acid. On the other hand, in EFA deficient diet group, the specific pattern was observed ; marked decrease of linoleic and arachidonic acid, marked increase of 5, 8, 11-eicosatrienoic acid, and moderate increase of palmitoleic acid. In any kinds of diet groups, qualitative changes of palmitic acid was not observed (Table 7, Fig. 3).

(3) Effect of Stress

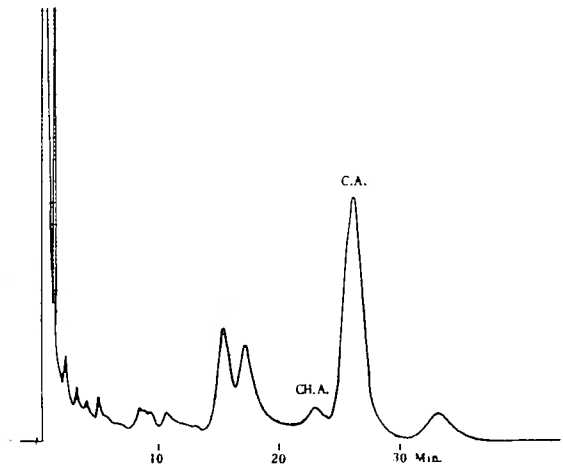
In this study, the intramuscular injection of ACTH-Z was used as one of stressors. Rats were injected daily 3.0 I.U. of 4 days or 1.0 I.U. of ACTH-Z for 14 days. The last injection was performed at one hour prior to the insertion of polyethylene tube. And

Fig.2 Gaschromatogram of Bile Acids

Standard Sample
D. A.: Deoxycholic Acid
CH. A.: Chenodeoxycholic Acid
C. A.: Cholic Acid



Rat Fed an EFA Diet



Rat Fed an EFA Deficient Diet

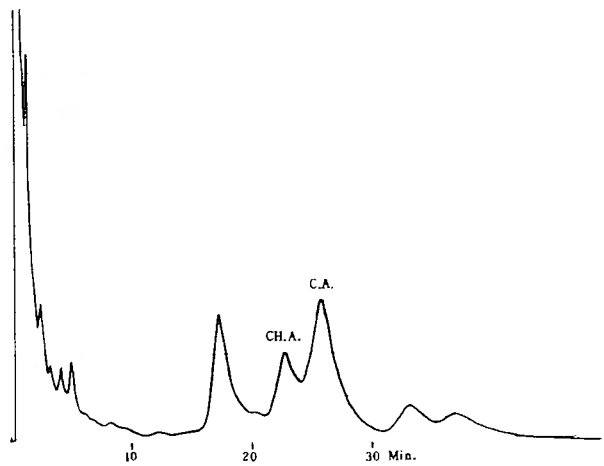


Table 6 Comparison between Composition of Total Fatty Acids and of Lecithin Composing Fatty Acids in the Same Bile of Rat

Fatty Acid	No. 1 Rat		No. 2 Rat		No. 3 Rat		No. 4 Rat	
	Total F. A.	Lecithin Composing F. A.	Total F. A.	Lecithin Composing F. A.	Total F. A.	Lecithin Composing F. A.	Total F. A.	Lecithin Composing F. A.
14 . 0	0.5*	0.8	0.4	0.6	0.3	0.9	0.6	1.0
15 : 0	0.2	0.6	0.2	0.6	0.3	0.5	0.7	0.8
16 : 0	39.2	40.3	45.0	43.7	34.3	31.0	31.0	31.0
16 : 1	3.4	3.5	4.6	3.9	3.2	2.3	2.9	3.2
17 : 0	0.5	0.5	0.4	0.1	0.6	0.6	0.6	0.6
18 : 0	6.9	7.3	6.8	7.1	10.4	11.6	8.2	9.6
18 : 1	12.0	12.1	12.7	13.6	9.7	10.3	9.2	10.0
18 : 2	28.6	27.0	23.8	23.9	23.3	25.0	27.7	27.9
18 : 3	trace	trace	trace	trace	trace	trace	trace	trace
20 : 3	0.5	0.7	0.4	0.1	1.0	0.3	0.5	0.2
20 : 4	7.9	7.5	5.8	6.1	16.9	14.7	15.7	13.0
22 : 4	trace	trace	trace	trace	trace	trace	trace	trace
22 : 6	trace	trace	trace	trace	trace	trace	trace	trace

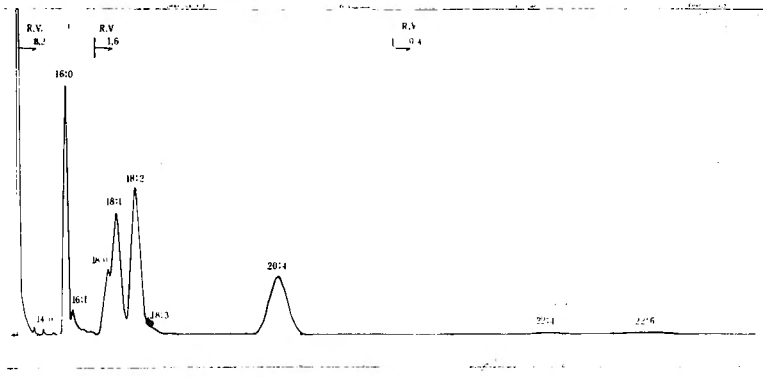
* : Percentage of total area of gas-liquid chromatographic elution diagram
No. 1 and No. 2 Rats fed standard diet
No. 3 and No. 4 Rats fed EFA diet

Table 7 Fatty Acid Composition and Amounts of Total Fatty Acids in Bile of Rats Fed Various Diets

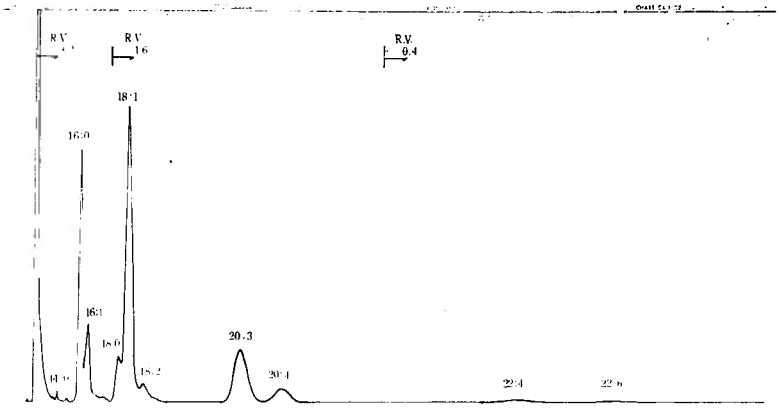
Fatty Acid	EFA Diet	EFA Deficient Diet	Vit. B_6 Deficient EFA Diet	Vit. B_6 Deficient EFA with Lard Diet	Mg Deficient EFA Diet
11 : 0	0.7±0.3** *	0.4±0.1	0.6±0.1	0.5±0.1	0.3±0.1
15 : 0	0.4±0.0	0.4±0.0	0.2±0.0	0.3±0.0	0.3±0.1
16 : 0	31.5±0.9	31.1±1.1	36.5±1.0	31.2±2.3	35.8±1.1
16 : 1	3.1±0.3	9.3±1.5	3.9±0.2	2.1±0.4	4.0±0.7
17 . 0	0.4±0.1	0.4±0.1	0.3±0.0	0.2±0.1	0.2±0.1
18 : 0	8.5±0.6	4.7±0.4	5.1±0.3	8.1±0.2	4.8±0.1
18 : 1	11.1±1.0	40.2±2.0	13.7±0.5	18.9±1.1	14.6±0.6
18 : 2	22.1±1.1	2.8±0.2	23.0±0.6	20.6±0.8	21.1±0.7
18 : 3	trace	trace	trace	trace	trace
20 : 3	trace	7.3±0.3	trace	trace	trace
20 : 4	18.5±0.8	3.0±0.5	16.5±0.3	18.2±1.8	17.7±0.6
22 : 4	trace	trace	trace	trace	trace
22 : 6	trace	trace	trace	trace	trace
Total Fatty Acids (mg/dl)	220.5±11.3**	181.5±7.5	214.5±13.7	161.3±27.2	205.3±18.9

* : Percentage of total area of gas-liquid chromatographic elution diagram
** : Standard error

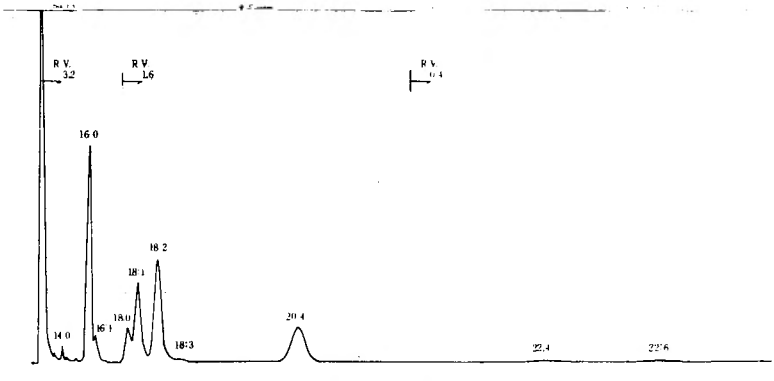
Fig. 3 Gaschromatogram of Fatty Acids in Bile of Rats Fed Various Dits



Rat Fed an EFA Diet



Rat Fed an EFA Deficient Diet



Rat Fed a Vit. B_6 Deficent EFA with Lard Diet

Table 8 Cholesterol and Bile Acids in Bile of Rats Fed Various Diets before and after Administration of ACTH-Z 3.0 I.U. for 4 Days or 1.0 I.U. for 14 Days

		Cholesterol (mg/dl)	Total Bile Acids (mg/ml)	Ratio of Dihydroxycholanic acid to Trihydroxycholanic Acid	Ratio of Total Bile Acids to Cholesterol
(1) EFA Diet	before	9.9±0.8*	1.15±0.30	0.43±0.10	42.8±7.6
	after[a)]	9.4±0.7	1.34±1.20	0.48±0.05	46.2±11.2
(2) EFA Deficient Diet	before	12.6±1.6	2.37±0.29	0.75±0.09	20.9±3.6
	after[a)]	13.0±1.8	2.08±0.25	0.60±0.07	15.9±0.9
(3) Vit. B_6 Deficient EFA Diet	before	12.8±0.6	1.91±0.25	0.93±0.12	15.5±2.3
	after[a)]	11.5±0.6	1.93±0.36	0.71±0.03	16.7±1.3
	after[b)]	12.9±0.8	1.63±0.09	0.71±0.09	12.7±0.0

(a) : After administration of ACTH-Z 3.0 I.U. for 4 days
(b) : After administration of ACTH-Z 1.0 I.U. for 14 days
* : Standard error

Table 9 Fatty Acid Composition and Amounts of Total Fatty Acids in Bile of Rats Fed Various Diets before and after Administration of ACTH-Z 3.0 I.U. for 4 Days or 1.0 I.U. for 14 Days

Fatty Acid	EFA Diet		EFA Deficient Diet		Vit. B_6 Deficient EFA Diet		
	before	after[a)]	before	after[a)]	before	after[a)]	after[b)]
14 : 0	0.7±0.3* **	0.5±0.0	0.6±0.1	0.6±0.1	0.6±0.1	0.4±0.0	0.5±0.1
15 : 0	0.4±0.0	0.3±0.1	0.4±0.0	0.3±0.1	0.2±0.0	0.2±0.0	0.3±0.1
16 : 0	31.5±0.9	33.2±2.2	31.1±1.1	29.6±1.1	36.5±1.0	37.1±1.0	38.7±0.9
16 : 1	3.1±0.3	2.9±0.3	9.3±1.5	11.9±0.9	3.9±0.2	2.2±0.2	3.2±0.2
17 : 0	0.4±0.1	0.4±0.1	0.4±0.1	0.7±0.2	0.3±0.0	0.2±0.0	0.2±0.0
18 : 0	8.5±0.6	10.3±1.3	4.7±0.4	5.3±0.8	5.1±0.3	7.3±0.6	7.3±0.6
18 : 1	11.1±1.0	13.0±0.7	40.2±2.0	37.3±1.4	13.7±0.5	11.8±0.4	13.5±0.8
18 : 2	22.1±1.1	20.0±1.9	2.8±0.2	4.3±0.8	23.0±0.6	22.9±1.4	19.3±2.8
18 : 3	trace	trace	trace	trace	trace	trace	trace
20 : 3	trace	trace	7.3±0.3	6.8±0.2	trace	trace	trace
20 : 4	18.5±0.8	17.5±1.9	3.0±0.5	3.1±0.5	16.5±0.3	18.1±0.8	16.7±0.8
22 : 4	trace	trace	trace	trace	trace	trace	trace
22 : 6	trace	trace	trace	trace	trace	trace	trace
Total Fatty Acids (mg/dl)	220.5±11.3**	194.3±13.8	184.5±7.5	123.4±13.6	211.9±13.7	205.1±22.1	233.1±26.6

(a) : After administration of ACTH-Z 3.0 I.U. for 4 days
(b) : After administration of ACTH-Z 1.0 I.U. for 14 days
* : Percentage of total area of gas-liquid chromatographic elution diagram
** : Standard error

then, the bile was collected aseptically during 24 hrs. and biochemically analyzed as described earlier. Each of groups consisted of 5 rats.

In EFA diet group, no significant change was observed. In EFA deficient diet group, total bile acids, B/C-ratio and total fatty acids decreased slightly. In Vit. B_6 deficient EFA diet group, no significant change was observed after the injection of ACTH-Z 3.0 I.U. for 4 days; however, after the injection of ACTH-Z 1.0 I.U. for 14 days, total bile acids and B/C-ratio decreased slightly. On the other hand, no significant changes on the composition of total fatty acids was observed in three groups after the injection of ACTH-Z (Table 8, 9).

It is considered that the stress will make a condition predisposing to gallstone formation in EFA deficient diet group and in Vit. B_6 deficient EFA diet group.

IV DISCUSSION

As stated in the introduction, nowadays the true initiating factor of gallstone formation is still obscure and many reports claiming that local factors (inflammation of gallbladder or biliary ducts, bile stasis, minimal damage of gallbladder and so on) play a primary role have been accepted commonly.

FUKUDA showed that adenocortical capacity and serum tetraenoic acid in the patients with gallstones decreased more than that in the control patients, and the same results were observed in the rats with EFA deficiency or its metabolic disturbance. This shows that systemic factors consisting of EFA deficiency or its metabolic disturbance will be important on gallstone formation. However, the detailed reports of the same opinion are few.

A colloid-biochemical unbalance of bile constituents being a direct cause of gallstone formation has been well known. The importance of the presence of cholesterol saturation in bile on cholesterol gallstone formation has beeen indicated by the fact that human bile was always saturated or almost completely saturated with cholesterol unlike to the bile of other animals, and also evidenced by solubility-test of human gallstones *in vivo* or *in vitro*. In this paper, cholesterol increased very slightly, but not significantly, in bile of EFA deficient rats and Vit. B_6 deficient EFA rats, and it was almost the same in amount in Vit. B_6 deficient EFA with lard rats as in control rats. Also, MARUYAMA reported that there was no statistical quantitative difference of cholesterol in bile between patients with gallstones and control patients. These facts show that the increased concentration of cholesterol in bile itself can not be a primary factor, especially on the experimental gallstone formation.

Total bile acids and B/C ratio have been indicated to be important for holding cholesterol soluble in bile. Recently, JOHNSTON and ISAKSSON have shown that phospholipid presenting in bile is also of importance. ISAKSSON reported that lecithin formed with part of the bile salts a stable complex lecithin bile salts system (L.B.S.) and cholesterol is more soluble in L.B.S. than it is in bile salts alone. In fact, L.B.S. and ratio of L.B.S. to cholesterol decreased in bile of patients with gallstones (ISAKSSON). More recenly many Japanese investigatrs, SHIMURA, YOSHIMUTA, and FURUSAWA, with an aid of paper-electrophoresis or on the colloid-chemical standpoint and so on, verified the same effect of L.B.S..

In this paper, a decrease in total bile acids, B/C-ratio and total fatty acids, namely lecithin, and an increase in Di/Tri-ratio were observed in bile of EFA deficient rats. MARUYAMA observed the same patterns in bile of patients with cholesterol gallstones when unassociated with both of bacterial infection and damage of liver function. SATŌ also reported an increase in B/C ratio and phospholipids in bile under the administration of unsaturated fatty acid in rabbits. SHIODA succeeded to produce experimental gallstones in a high ratio in EFA deficient golden hamsters. By these facts, the importance of EFA deficiency on the experimental gallstone formation was verified.

BLOCH reported that bile acids were end-metabolite of cholesterol in the liver and thereafter, with radio active isotope, BYERS, SIPERSTEIN, and BERGSTRÖM clarified its metabolic process.

HIRANO observed that in the liver of EFA deficient rats, EFA such as linoleic, arachidonic and 4, 7, 10, 13, 16-docosapentaenoic acid, which formed esters with cholesterol and activated cholesterol metabolism, decreased and then, the fatty acids of oleic and palmitoleic acid series, which were synthesized in the body and might not be able to activate cholesterol metabolism if cholesterol combined with these fatty acids, increased. In other words, the activation of cholesterol metabolism occurs in the liver of EFA deficient rats, and it caused in bile a decrease in total bile acids and B/C-ratio, and an increase in Di/Tri-ratio, which shows metabolic disturbance among mono-, di- and trihydroxycholanic acid. Besides, synthetic capacity of lecithin in the liver and absolute amounts of lecithin in bile decrease. And then, cholesterol gallstones in experimental animals will be formed.

However, it is sure that deficiency itself in EFA is not the true cause in cholesterol gallstone formation in human beings. Because, there are two different points, though the biochemical patterns in liver and bile of EFA deficient rats are almost similar to those in patients with gallstones.

(1) The data in this paper showed that there were significant decrease in linoleic and arachidonic acid with significant increase in oleic and 5, 8, 11-eicosatrienoic acid in percentage in EFA deficient rats. However, no significant defference of fatty acid composition in bile was observed between control patients and patients with cholesterol gallstones (BLOMSTRAND and MARUYAMA). In other words, the fatty acid composition in bile is significantly different in patients with cholesterol gallstones from that in EFA deficient rats. On the other hand, NISHIMURA and WATANABE reported that a decrease in linoleic acid, an increase in oleic acid and a decrease in ratio of linoleic acid to oleic acid were specific changes observed in bile of patients with gallstones.

(2) In spite of the decrease in percentage as well as in absolute amount of linoleic acid in the liver of EFA deficient rats, the decrease was not observed or it was very slight in the liver of patients with cholesterol gallstones (HIRANO).

In other words, as the patients with gallstones intake linoleic acid sufficiently from the vegetable oils and other food stuffs, the deficiency in linoleic acid does not occur. These data indicate that metabolic disturbance in EFA is present as a main factor of cholesterol gallstones.

Regarding metabolic disturbance in EFA, a deficiency in Vit. B_6 is considered in

first place. Because, the important role of Vit. B_6 in fatty acid metabolism, especially in conversion of linoleate to arachidonate, has been demonstrated by many investigators.

In this paper, using rats fed a diet containing enough linoleic acid under a relatively deficient state in Vit. B_6, the bile constituents were very similar to those of the patients with cholesterol gallstones, except for the absence of decreased total fatty acids (i.e. lecithin). In this case, the spectrum of lecithin composing fatty acids resembled that in control rats unlike to that in EFA deficient rats. Also, HIRANO observed the same patterns in the liver as in patients with gallstones, except for the increase in total and esterified cholesterol.

Therefore, when 10% of lard and 5% of sesame oil were added to the Vit. B_6 deficient diet, there appeared a decrease in total bile acids, B/C-ratio and total fatty acids (i.e. lecithin), an increase in Di/Tri-ratio, and the same composition of lecithin composing fatty acids as in control rats. These patterns were exactly similar to those of patients with cholesterol gallstones when compared to control patients. Also, HIRANO demonstrated in the liver of rats fed a this diet that qualitative and quantitative changes in cholesterol and fatty acids were similar to those in patients with cholesterol gallstones.

MARUYAMA verified the importance of Vit. B_6 in the clinical experiment; when patients were treated with sōya-lecithin containing excessive linoleic acid and Vit. B_6, B/C-ratio in drainaged bile showed a great increase as compared with non-treated.

On the other hand, it has been pointed out that a large absorption of saturated fatty acids brought Vit. B_6 deficiency, and the biosynthesis of Vit. B_6 was counteracted by the administration of sulfur amino acids, especially methionine and cystine. In those who are used to take ample proteins and animal fats (mainly consisting of saturated fatty acids), a deficieny in Vit. B_6 and an increase of cholesterol supply develope. As a matter of fact, it is informed that cholesterol gallstones are observed frequently in European and American who are used to take animal fat and protein in larger quantity than in Jananese. It is considered that the frequent occurrence of cholesterol gallstones in Japanese recently is due to an increase of taking animal fat and protein.

MARUYAMA reported that bile acids in the liver showed a manifest decrease under the administration of ACTH-Z in Vit. B_6 deficient rats. The data in this paper showed that the adminstration of ACTH-Z brought more marked decrease of total bile acids and B/C-ratio than they were at rest, in EFA deficient rats and in Vit. B_6 deficient EFA rats. From these experiments, it is considered that the repeated exposure to stress will promote a condition predisposing to gallstone disease.

As EFA is also contained in the steroid hormone secreting organs, for example adrenals and ovaries, the hypofunction in these organs may occur when the metabolism of EFA is disturbed. It has been reported that cholesterol gallstones occur more frequently in women than in men, especially in post-climacteric or in obese delivery experienced women. Thus, it can not be neglected that the function of the endocrine system may be concerned with gallstone formation. SHIMURA reported that bile constituents in the terminal stage of pregnancy were similar to those in patients with gallstones. According to WACHSTEIN, PAL-PO_4 levels of circulating leucocytes were significanly lower in pregnant women than in no-pregnant women. This exhibits that a relative Vit. B_6 deficiency exists

during pregnancy. These reports suggest the positive relation between pregnancy and gallstone formation.

Briefly saying on the main process of cholesterol gallstone formation, the relative deficiency in Vit. B_6 and the metabolic disturbance in EFA may be primary and result in (1) the disturbance in cholesterol activation, the disturbed biosynthesis of bile acids from cholesterol in the liver with accumulation of cholesterol, the decrease in total bile acids and B/C-ratio, and the increase in Di/Tri-ratio in bile, and (2) diminution of lecithin in bile. These pathological changes in bile and liver play an important role in forming cholesterol gallstones. When once gallstone is formed, it may cause a secondary inflammation in the gallbladder and bile ducts. It is considered that the various changes following the secondary inflammation will damage the colloidal stability of bile.

Regarding the initiating factors of bilirubin-pigment gallstone formation, the local factors such as the intensified activity of β-glucuronidase, the nucleal formation of gallstones by parasites, the changes of protein, bile salts and calcium concentration in bile, and so on, have been discussed. However, an idea that systemic factor has primary significance might be inferred in the light of cholesterol gallstone formation process. It is reported that an increase in total calcium and calcium deposition in the tissues occurred in magnesium deficient rats. Moreover, the significance of magnesium deficiency in regard to renal stone formation has been demonstrated.

The data in this paper showed very slight increase in cholesterol and Di/Tri-ratio, moderated decrease in total bile acids and B/C-ratio, and a tendency to decrease in total fatty acids (i. e. lecithin) in the bile of magnesium deficient rats. According to these data and reports that there is significantly negative mutuality between cholesterol and magnesium concentration in serum of various races and that dietary deficiency in magnesium accelerate an occurrance of experimental atherosclerosis, it may be suggested that dietary deficiency in magnesium bring out the disturbance of cholesterol metabolism or the disturbance of fatty acid metabolism, especially that of EFA.

However, TANIMURA and HASHIMOTO failed to observe a significant difference between calcium concentration in bile of magnesium deficient rats and that of control rats. In other words, the significance of magnesium deficiency on gallstone formation is not assured.

V SUMMARY AND CONCLUSION

The effects of EFA deficiency and its metabolic disturbances on the bile constituents of rats were studied under various diets, such as EFA diet (taken as control), EFA deficient diet, Vit. B_6 deficient EFA diet, Vit. B_6 deficient EFA with lard diet, and magnesium deficient EFA diet.

(1) Regarding cholesterol amounts in bile, moderate increase in other rats than in Vit. B_6 deficient EFA with lard rats was observed. But as these changes were not statistically significant, EFA deficiency and its metabolic disturbance may not be related significantly to cholesterol amounts in bile of rats.

(2) The significant decrease in total bile acids and B/C-ratio, and significant increase in Di/Tri-ratio were observed in other rats than in magnesium deficient EFA rats. In latter rats, moderate decrease in total bile acids and B/C-ratio, and slight increase in

Di/Tri-ratio were observed. It is a specific point that these similar changes as in bile of patients with cholesterol gallstones were observed not only in EFA deficient rats but also in EFA sufficient rats with Vit. B_6 or magnesium deficiency.

(3) Absolute amounts of total fatty acids, i.e. of lecithin, decreased remarkably in EFA deficient rats and in Vit. B_6 deficient EFA with lard rats, and decreased very slightly in other rats.

(4) Main lecithin composing fatty acids were palmitic, oleic, linoleic and arachidonic acid in other rats than EFA deficient rats. In EFA deficient rats were observed the significant decrease in linoleic and arachidonic acid, and the significant increase in palmitoleic, oleic and 5, 8, 11-eicosatrienoic acid, which did not occur in bile of patients with cholesterol gallstones.

(5) From these results, it may be justified to consider that EFA deficiency and its metabolic desturbance with Vit. B_6 deficiency disturb cholesterol metabolism toward bile acids and metabolic process of bile acids themselves. And then metabolic disturbance in EFA, especially with Vit. B_6 deficiency, may play a primary role on cholesterol gallstone formation. The local factors, for example inflammation of gallbladder or biliary ducts and bile stasis, may be secondary.

(6) The exposure to repeated stresses will give more suitable changes of bile constituents for cholesterol gallstone formation.

(7) A role of magnesium on gallstone formation is not yet conclusive.

The author wishes to express his sincere gratitude to Assist. Prof. Dr. Y. HIKASA for his helpful suggestions and kind guidences throughout this study. The author also thanks to Dr. I. MARUYAMA for his cooperation. (The gist of this article was reported at the 63rd and 64th General Meeting of Japanese Surgical Society)

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和文抄録

コレステロール系胆石生成に関する実験的研究，特に不可欠脂酸及びピリドキシンの胆汁組成に及ぼす影響について

京都大学医学部外科学教室第2講座（指導：木村忠司教授）

吉永道生

胆石の成因については，Naunyn の炎症説及び Aschoff－Bacmeister の非炎症性胆汁鬱滞説などを始めとして，更には，膠質化学的立場から説明を加えるなど色々の報告がなされているが，決定的な説明はなく，局所因子を第一義的なものとする考え方が強い．一方日笠は不可欠脂酸（EFA）と Adrenocortical Capacity との間に密接な関係があることを認め，コレステロールから副腎皮質ホルモンの合成には EFA，特に Tetraenoic acid がコレステロールとエステル結合することが必要であると報告した．また，福田は胆石症患者の Adrenocortical Capacity が低下していることを認めた．

丸山はコレステロール系胆石症患者の胆汁では総胆汁酸量及びレシチン量の減少，総胆汁酸対コレステロール比の低下，Dihydroxycholan 酸対 Trihydroxycholan 酸比の上昇があることを証明している．而も，胆汁酸はコレステロールの代謝産物の一つであり，コレステロールから胆汁酸への代謝過程に EFA が重要な役目をもつことが類推される．そこで，脂質殊に EFA の有無及び脂質代謝に密接な関係を有する Vit. B_6 の有無が胆汁組成にどのような影響を及ぼすかを実験的に検討した．即ち，ウイスター系雄性ラットを5群—EFA 投与食群（対照群とする），EFA 欠乏食群，Vit. B_6 欠乏 EFA 投与食群，Vit. B_6 欠乏 EFA・ラード投与食群——にわけて2～3ヵ月に亘り飼育し，総胆管にチューブを挿入，24時間肝胆汁を採取し生化学的分析を行い，次の様な結果を得た．

(1) コレステロールは Vit. B_6 欠乏 EFA・ラード投与食群を除く他群では軽度の増加を示したが，いづれも有意の差を認めなかつた．

(2) EFA 欠乏食群，Vit. B_6 欠乏 EFA 投与食群及び Vit. B_6 欠乏 EFA・ラード投与食群に於いて，総胆汁酸量の減少，総胆汁酸対コレステロール比の低下及び Dihydroxycholan 酸対 Trihydroxycholan 酸比の上昇を推計学的にも判然と認め得た．即ち，EFA 欠乏食ラットのみならず EFA が仮令十分に投与されていても Vit. B_6 の欠乏を伴つたラットに於いては，胆石症患者のそれと同様の変化を認め得たということになる．

(3) 総脂酸量即ちレシチン量は EFA 欠乏食群及び Vit. B_6 欠乏 EFA・ラード投与食群で著明に，Vit. B_6 欠乏 EFA 投与食群では軽度に減少した．

(4) レシチンを構成する脂酸は，EFA 欠乏食群では Linol 酸及び Arachidon 酸の減少，Palmitolein 酸，Olein 酸及び 5, 8, 11－Eicosatrien 酸の著明な増加を認め，胆石症患者のそれと全く異なつた像を示した．一方，Vit. B_6 欠乏 EFA 投与食群及び Vit. B_6 欠乏 EFA・ラード投与食群では主な脂酸は Palmitin 酸，Olein 酸，Linol 酸及び Arachidon 酸で，対照群と類似の像を示した．

(5) 以上の実験結果から，EFA の欠乏及び相対的 Vit. B_6 欠乏を伴つた脂質代謝障害殊に EFA の代謝障害はコレステロールから胆汁酸への代謝異常及び胆汁酸自体の代謝異常と，レシチンの合成能低下を惹起することが推測される．更に，平野が示すように胆石症患者では肝臓中 Linol 酸の減少を認めないこと，動物性脂質の過剰摂取や高蛋白食が Vit. B_6 量の減少を惹起すること，Vit. B_6 は腸内細菌によつて合成されうることなどを考え合せると，臨床的には相対的 Vit. B_6 欠乏による脂質殊に EFA 代謝異常がコレステロール系胆石生成に密接な関係を有すると推測される．このことは，胆石生成機転において全身的要因が第一義的役割をなすことを示している．

(6) ACTH 投与などのストレス負荷は，コレステロール系胆石生成の好条件を胆汁組成に与える．

(7) マグネシウム欠乏 EFA 投与食群で，総胆汁酸量の中等度減少をみとめ，マグネシウム欠乏がコレステロールから胆汁酸への代謝過程に影響を与えることは推測出来るが，胆石成因との関係については明らかにすることは出来なかつた．